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Evaluation of Immunological Responses in Patients with Ovarian Cancer Treated with the Anti-idiotype Vaccine ACA125 by Determination of Intracellular Cytokines—A Preliminary Report

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ABSTRACT

In a first clinical trial, 45 patients with advanced ovarian carcinoma and recurrences were treated with the murine monoclonal anti-idiotypic antibody (Ab2) designated ACA125 for active immunotherapy. The monoclonal antibody (MAb) ACA125 mimics a specific epitope of the tumor-associated antigen CA125 expressed by most malignant ovarian tumors. Patients with CA125-positive tumors are immunologically tolerant to CA125, which could be overcome by the use of an anti-idiotypic antibody as a surrogate for the tumor antigen CA125. An immunological response to the anti-idiotype ACA125 in these patients was associated with a statistically significant survival prolongation. Humoral immunity to ACA125 was assessed by induction of anti-anti-idiotypic antibodies (Ab3) directed against CA125. Using flow cytometric detection methods we observe alterations of the intracellular cytokines IFN-γ, IL-2, and IL-4 at the single-cell level during the course of immunization. There was a strong increase of intracellular IFN-γ and IL-2 characteristic for a Th1 cell type immune response after treatment with ACA125. A delayed induction of Th2 type response, which promotes antibody-mediated immunity by B cells, could also be detected. The understanding of the kinetics of Th1 and Th2 responses could be important to improve treatment schedules for effective immunotherapy with anti-idiotype vaccines.

INTRODUCTION

OVARIAN CANCER represents the fourth most common cause of death in women and the leading cause of gynecological cancer death in the United States.[1] Approximately 80% of the ovarian cancer cases will be detected in advanced stages and 60% of all ovarian cancer patients suffer from tumor masses larger than 10 cm at the time of primary therapy. Whereas early tumor stages show survival rates between 80% (for stage 1) and 20–45% (for stage 2), 5-year survival rates for advanced stages of disease (FIGO stages 3 and 4) with spread of tumor masses in the peritoneal cavity and/or lymph node metastases (3a–c) or distant metastases like lung or intrahepatic metastases (4) are not higher than 10%.[1]

Despite an increased extent of radical surgery and intensification of polychemotherapy, cure rates could only be marginally increased. Therefore, the establishment of new therapeutic strategies including adjuvant immunotherapy should be considered.[2]

A new concept of active immunotherapy of ovarian carcinoma implicates the transformation of a critical epitope of the tumor-associated antigen CA125 expressed in over 80% of ovarian cancer into idiotypic determinants of an antibody; thus breaking the immunological tolerance to this antigen. According to the idiotypic network approach by N. Jerne, such anti-idiotypic antibodies (Ab2) that bind to the antigen-combining site of anti-tumor antibodies (idiotypic antibody, Ab1) functionally imitate the tumor-associated antigen defined by the Ab1.[3] Immunization with an anti-idiotypic antibody leads to the induction of anti-anti-idiotypic antibodies (Ab3) directed against the tumor-antigen.[4–6] In addition, Ab2 themselves, when processed with major histocompatibility complex (MHC)

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molecules on antigen-presenting cells (APC) after the formation of so-called "idiopeptides," could generate an antitumoral cellular immune response based on an undirected natural killer (NK)-cell activity or on induction of specifically acting cytotoxic T lymphocytes.[7] We have generated a murine monoclonal anti-idiotypic antibody, named ACA125, which was shown to be the internal image of the CA125 antigen.[8,9]

In a first clinical trial, 45 patients with advanced ovarian cancer and recurrences were treated with the anti-idiotype vaccine ACA125. Patients with high amounts of specific anti-anti-idiotypic antibodies (Ab3) directed against CA125 have a significant improved survival. This positive correlation between humoral immunity indicated by the induction of Ab3 and survival of cancer patients could also be shown in anti-idiotype vaccination trials in cases with colorectal cancer[10,11] and melanoma.[12]

In contrast, the description of a specific anti-tumoral cellular immune response seems to be difficult due to a lack of reliable detection methods. We were therefore searching for alternative parameters to measure the activation of the humoral and cellular defense lines in the case of immunization with anti-idiotypic antibodies. The type of immune response that develops after antigen (or anti-idiotype) priming may be determined by the appearance of two $CD4^+$ T-helper cell phenotypes termed Th1 and Th2. These Th1 and Th2 subsets can be functionally distinguished by their cytokine profile.[13] Th1 cells produce IL-2, IFN-γ, and lymphotoxins that are involved in cell-mediated cytotoxic functions, that is, delayed-type-hypersensitivity (DTH) response. In contrast, the Th2 subset produces IL-4, IL-5, IL-9, IL-10, and IL-13 and promotes antibody production by B cells.[14,15] The determination of intracellular Th1 and Th2 cytokines at the single T-cell level can be used to characterize indirectely the outcome as well as the kinetics of humoral and cellular responses to an anti-idiotypic stimulus.

In the following report we describe the intracellular detection of the Th1 cytokines IFN-γ and the IL-2 and Th2 cytokine IL-4 at several time points during the course of immunization with the anti-idiotype ACA125 in seven ovarian cancer patients.

MATERIALS AND METHODS

Patients

In the present study 7 patients with advanced ovarian carcinoma and recurrences were monitored for IFN-γ, IL-2, and IL-4 production at the single T-cell level during immunotherapy with the anti-idiotypic vaccine ACA125. The treatment schedule implicates four initial vaccinations with 2 mg alum-precipitated complete anti-idiotypic antibody ACA125 every second week followed by immunizations in 4-week intervals.[9]

As we wanted to study whether the immunization status influences the cytokine response, we selected patients who had received a different number of ACA125 applications: patient D.K. 3, H.E. 4, A.B. 12, G.L.-R. 13, A. Bü. 14, M.Pl. 20, and M.P. 33 immunizations. Three healthy females were taken as control to exclude normal physiological changes of IFN-γ, IL-2, and IL-4 production in the group of vaccinated ovarian cancer patients. Additional controls, that is, ACA125-treated healthy probands were not included due to ethic considerations.

Peripheral blood samples (ammonium-heparinat) were taken at different timepoints during one course of immunization (Day 0 = day of immunization; Day 3, 6, 9, 13, 16, and 32 = next immunization).

Antibodies for flow cytometry

The following MAbs (Becton-Dickinson, Heidelberg, Germany) were used for intracellular staining of cytokines: anti-IFN-γ (FITC, 25723.11), anti-IL-4 (PE, 3010.211), and anti-IL-2 (FITC, 5344.111). Surface phenotyping of lymphocytes was determined by anti-CD3 (FITC, PerCP, SK7), anti-CD4 (FITC, PE, SK3), anti-CD8 (FITC, PE, SK1), anti-CD16+56 (FITC, PE, NCAM 16.2 + P3X), anti-CD19 (PE, 4G7), anti-HLA-DR (PE, L243), and anti-CD69 (PE, L78; Becton-Dickinson, Heidelberg, Germany).

Analysis of intracellular cytokines

Staining of intracellular cytokines was performed as described previously.[16,17] Briefly, peripheral blood was diluted with RMPI (1:2) and stimulated with PMA (15 ng/mL) and ionomycin (1 μg/mL) in the presence of the inhibitor of intracellular transport Brefeldin A (10 μg/mL) for 5 h, at 37°C and 5% CO_2. Cells were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde and permeabilized with 0.1% Saponin (an der Grub). After incubation with anti-cytokine antibodies, cells were washed and surface staining was performed with anti-CD3 antibody. T cells were analyzed at the single cell level by three-color flow cytometry (FACSCalibur, Becton Dickinson) including acquisition of 2000 events in the lymphocyte-gate.

Detection of early activation markers (CD69 expression)

CD69-expression on lymphocyte subsets was measured in peripheral blood samples after stimulation with PMA (15 ng/mL) and ionomycin (1 μg/mL) for 5 h, at 37°C and 5% CO_2. Surface staining and flow cytometric analysis were performed as described elsewhere.[18]

Immunophenotyping of lymphocytes

The following lymphocyte subsets were determined in erythrocyte-lysed whole blood by flow cytometric analysis as described above: T ($CD3^+$) lymphocytes, B (CD19) lymphocytes; helper T ($CD3^+CD4^+$) lymphocytes, cytotoxic T ($CD3^+CD8^+$) lymphocytes and NK ($CD3^-CD16^+56^+$) lymphocytes.

Detection of human anti-mouse antibodies (HAMA)

Human anti-mouse antibodies (HAMA) in sera of patients including allo- and isotypic-antibodies were measured using a commercial available HAMA-enzyme-linked immunoadsorbent assay (ELISA) (medac, Hamburg, Germany).

Detection of specific anti-anti-idiotypic antibodies (Ab3)

The induction of anti-anti-idiotypic antibodies (Ab3) in sera was measured as described previously.[8] Briefly, microtiter plates were coated with anti-idiotype ACA125 $F(ab)_2$ and in-

cubated with patients' sera. After addition of the complete ACA125, immune complexes were detected by rabbit-anti-mouse-IgG-POD-conjugate (Fc-specific, Dianova).

RESULTS

The kinetics of humoral and cellular immune responses of seven ovarian cancer patients with different cycles of anti-idiotype vaccination were monitored at several timepoints during one course of immunization (4 weeks), including one healthy female as normal control. Two patients in this study were suffering from additional inflammatory diseases during the course of immunization, so that intracellular cytokine production could not be cleary correlated to anti-idiotype vaccination.

HAMA and Ab3 response, immunophenotyping of lymphocytes, intracellular cytokines (IFN-γ, IL-2, IL-4), and early activation markers (CD69) were measured to describe the immunological effects of ACA125 treatment.

Humoral responses to the murine anti-idiotype ACA125

The development of a HAMA and a specific anti-anti-idiotypic immune response induced by immunization with the anti-idiotype ACA125 was assessed by testing patients' sera at Day 0 (day of immunization), 3, 6, 9, 13, 16, and 32 (day of next immunization).

A close association between the number of anti-idiotype application and increasing titers of HAMA- and Ab3-antibodies could be observed in all seven patients. Patient H.E. showed elevating concentrations of HAMA, including anti-iso-allotypic antibodies, and of specific Ab3 against CA125 from Day 0 to 9 (HAMA: 600 ng/mL; A3: 7325 ng/mL arb. Units), followed by a slight decrease from Day 16 to 32 (Fig. 1). In contrast, patient M.P., who had received much more cycles of immunization, developed a steady state of humoral immunity without significant changes of HAMA and Ab3 concentrations (Fig. 2). Additionally, HAMA and Ab3 titers were higher in comparison to patient H.E.

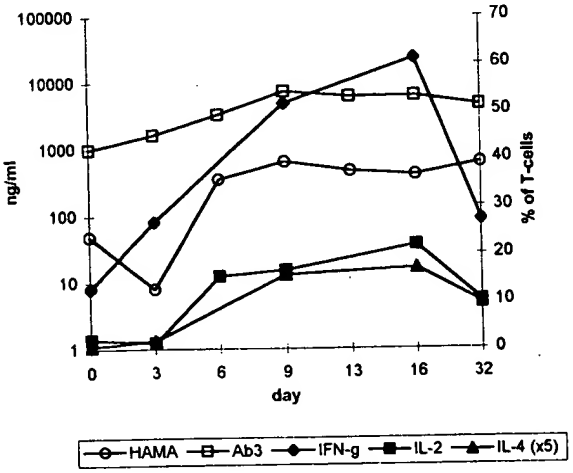

FIG. 1. Humoral and cellular immune responses to the anti-idiotypic antibody ACA125 in patient H.E. during one course of immunization. HAMA response (ng/mL) and specific anti-anti-idiotypic antibodies Ab3 (ng/mL arb. units) were detected in patients sera (logarithmic scale). Intracellular cytokines IFN-γ, IL-2, and IL-4 were detected in lysed whole blood of patient H.E.

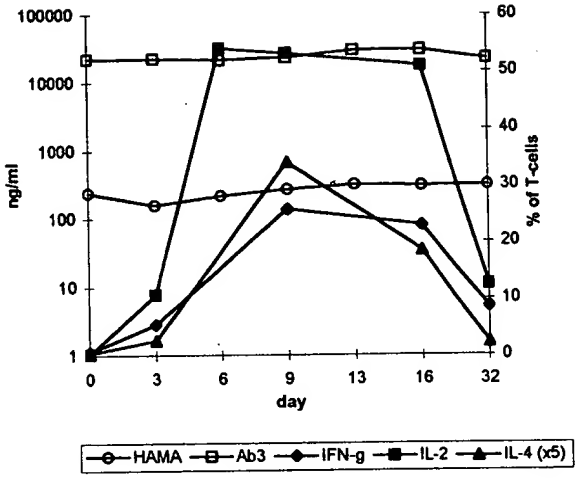

FIG. 2. Humoral and cellular immune responses to the anti-idiotypic antibody ACA125 in patient M.P. during one course of immunization. HAMA response (ng/mL) and specific anti-anti-idiotypic antibodies Ab3 (ng/mL arb. units) were detected in patients' sera (logarithmic scale). Intracellular cytokines IFN-γ, IL-2, and IL-4 were detected in lysed whole blood of patient M.P.

Intracellular cytokines IFN-γ, IL-2 and IL-4

The percentage of intracellular IFN-γ, IL-2, and IL-4 production by T lymphocytes were analyzed in peripheral blood samples at time points indicated above.

There was a remarkable increase of Th1 cytokines IFN-γ and IL-2 detectable from Day 0 to 16 (IFN-γ: 12.7 to 61.2%; IL-2; 2.0 to 21.0%) in patient H.E. The production of these cytokines was reduced until the day of next immunization (Fig. 1). The kinetics of Th2-specific cytokine showed a delayed increase of IL-4 in comparison to Th1 cytokines. The overall amount was lower due to a general low IL-4 production. Additionally Th1 and Th2 cytokine amounts correlates with HAMA and Ab3 concentrations measured in this patient.

In patient M.P. intracellular Th1 and Th2 cytokine production was similar to patient H.E. with a maximum at Day 6 and 9 (IFN-γ: 54%; IL-2: 25.7%; IL-4: 6.8%). No correlation between Th1/Th2 cytokines and HAMA/Ab3 response could be found in this patient.

Similar kinetics of Th1 and Th2 cytokine induction by anti-idiotype vaccination could be proven in all patients, but not in the healthy controls without anti-idiotype vaccination (IFN-γ: 14.3 ± 6.8%; IL-2: 30.4 ± 15.8%; IL-4: 1.0 ± 0.8% of T cells).

Immunophenotyping of lymphocytes

Patients lymphocytes were phenotyped according to their characteristic surface markers by flow cytometry during the

TABLE 1. IMMUNOPHENOTYPING OF LYMPHOCYTES FROM LYSED WHOLE BLOOD OF ACA125-VACCINATED PATIENTS DURING ONE COURSE OF IMMUNIZATION. MEAN VALUES AND RANGES (IN DASHES) OF ALL PATIENTS AT DIFFERENT TIMEPOINTS ARE INDICATED

	Day 0	*Day 3*	*Day 6*	*Day 9*	*Day 16*	*Day 24*	*Day 32*
B cells (CD3−/19+) % of Lymph	9.6 (5.0–16.0)	8.9 (5.0–12.0)	9.1 (5.0–13.0)	8.8 (5.0–12.0)	9.0 (4.0–14.0)	9.1 (4.0–14.0)	8.5 (5.0–17.0)
Tc cells (CD3+/8+) % of Lymph	33.0 (28.0–39.0)	31.8 (25.0–36.0)	31.6 (26.0–41.0)	32.6 (27.0–41.0)	31.8 (25.0–38.0)	33.4 (27.0–44.0)	35.1 (26.0–45.0)
Th cells (CD3+/4+) % of Lymph	38.8 (29.0–42.0)	38.0 (29.0–43.0)	39.0 (28.0–45.0)	41.6 (27.0–54.0)	41.2 (33.0–45.0)	40.4 (32.0–44.0)	39.2 (29.0–47.0)
NK cells (CD3−/16+56+) % of Lymph	19.2 (16.0–22.0)	18.6 (15.0–26.0)	19.0 (16.0–22.0)	18.2 (16.0–21.0)	20.8 (17.0–27.0)	20.0 (16.0–23.0)	20.2 (16.0–29.0)
Monocytes (CD3−/14+) % of Leuco	6.8 (4.0–10.0)	6.0 (4.0–8.0)	6.3 (4.0–7.0)	6.0 (3.0–8.0)	5.8 (5.0–9.0)	5.9 (5.0–9.0)	6.0 (4.0–9.0)

course of immunization: B cells (CD3−/CD19+), T cells (CD3+), activated T cells (CD3+/HLADR+), Th cells (CD3+/CD4+), Tc cells (CD3+/CD8+), and NK cells (CD3−/CD16+56+).

There were no significant changes detectable in the individual patients' subtype composition in association with ACA125 application. The mean values are given in Table 1.

Early activation markers (CD69+) expressed by Th, Tc, and NK cells

The CD69 cell surface marker is expressed within 3 days after a stimulus and indicates an early state of activation of a specific lymphocyte subtype. CD69 expression restricted to CD3/CD4+ Th cells, CD3/CD8+ Tc cells, CD3−/CD16+56+ NK cells were measured throughout the course of immunization using flow cytometry. Surprisingly, no significant activation of Th cells could be detected in all patients and NK cells were slightly stimulated over the first 6 days after vaccination only in patient M.P. An increase of CD69 expression of cytotoxic T cells from Day 0 to 3 appeared merely in two patients (Table 2). Because of the low CD69 expression values, these results cannot clearly demonstrate an early activation due to a anti-idiotypic stimulus, and could otherwise be interpreted as baseline expression.

DISCUSSION

The present study was conducted to assess the efficiency of the anti-idiotypic MAb ACA125, which functionally imitates the tumor-associated antigen CA125 to induce antitumoral immune responses in patients with advanced ovarian cancer. Whereas the induction of humoral immunity in patients treated with ACA125 vaccine could be sufficiently demonstrated by generation of HAMA and specific anti-anti-idiotypic antibodies (Ab3) directed against CA125,[9] we were searching for new parameters to describe and understand activation processes in cellular immunity.

Thus, seven patients immunized at various cycles of anti-idiotype immunotherapy were monitored for changes in the production of intracellular cytokines IFN-γ, IL-2, and IL-4,[16] early activation events (CD69 expression)[18] and lymphocyte subtype composition over a period of 4 weeks. In all patients an expansion of cellular and humoral immune responses to the anti-idiotypic stimulus could be detected by increasing production of Th1-(IFN-γ, IL-2) and Th2-cytokines (IL-4). Intracellular IL-4 production by Th2 cells develops 3 days later in the course of immunization, but the IL-4 values are lower in comparison to IFN-γ and IL-2. Possibly, stimulation with Ionomycin/PMA favors Th1 cytokine production and is not optimal for IL-4 production. These findings suggest an initial cellular immune response followed by a delayed humoral reaction

TABLE 2. EARLY ACTIVATION MARKERS (CD69+) EXPRESSED ON TH, TC, AND NK CELLS BY ACA125-IMMUNIZED PATIENTS DURING ONE COURSE OF VACCINATION. MEAN VALUES AND RANGES (IN DASHES) OF ALL PATIENTS AT DIFFERENT TIMEPOINTS ARE INDICATED

	Day 0	*Day 3*	*Day 6*	*Day 9*	*Day 16*	*Day 24*	*Day 32*
CD4+/CD69+ % of Th cells	6.8 (1.3–11.0)	7.5 (1.4–9.0)	6.0 (0.7–10.2)	8.9 (1.2–15.4)	6.4 (1.8–11.2)	6.2 (1.4–9.3)	8.9 (1.3–19.6)
CD8+/CD69+ % of Tc cells	5.1 (1.6–8.2)	6.4 (2.6–9.1)	5.5 (1.5–7.6)	5.7 (1.6–9.3)	3.4 (1.3–6.5)	2.9 (0.7–6.2)	5.9 (1.6–9.5)
CD16+/56+CD56 % of NK cells	8.6 (0.6–21.5)	9.0 (0.6–20.8)	9.4 (0.9–24.1)	10.4 (0.9–32.2)	8.4 (0.8–20.9)	8.0 (0.9–24.6)	7.3 (1.4–21.2)

mediated by Th2 cells. In nearly all patients the production of intracellular cytokines is down-regulated before starting next immunization cycle. Based on this observation it can be concluded that the existing treatment schedule (4 weeks between vaccinations) fits with the kinetics of Th1 and Th2 immune responses. Although it was difficult to find appropriate controls, it could be shown that the physiological ranges of IFN-γ, IL-2, and IL-4 production in normal females without vaccination was different, so that Th1/Th2 responses in the patients could be clearly associated to anti-idiotypic vaccination.

Additionally, there is a positive correlation between HAMA- and anti-anti-idiotypic antibody (Ab3) concentrations and Th1/Th2 cytokines in patient H.E. who received the fourth immunization. Otherwise, with increasing cycles of vaccination the patients establish a steady state of HAMA and Ab3 titers (as shown in patient M.P.). The increase of Th2 cytokine IL-4 could be responsible to maintain this high level of antitumoral antibody production. Immunophenotyping of patients' lymphocytes shows no significant alterations of the distinct subsets, which could be associated with an effect of anti-idiotypic vaccination. Co-stimulatory effects of monocytes on cytokine production could be excluded, because no changes of the monocyte population could be detected.

Early activation tendencies in Tc and NK cells as measured by CD69 expression could be seen in all patients during the first 6 days after immunization. Whether these events depend on an anti-idiotypic stimulus or represent a baseline CD69 expression, as detectable in healthy normals,[18] due to ongoing stimulation of varied numbers of T and NK cells, could not be shown. One explanation could be the rare frequencies of specific anti-idiotypic T-cell clones,[7] as only these cells should be capable of demonstrating activation at an early stage following anti-idiotypic stimulation. With increasing cycles of immunizations, the specific anti-idiotypic T-cell clones should raise in these patients indicated by expected higher CD69 expression. Indeed, patient M.P. who received 33 ACA125 vaccinations expresses higher levels of CD69 markers on Tc, Th, and NK cells compared to patients at the beginning of immunotherapy (e.g., patient H.E.). Our results show that CD69 as well as HLA-DR expression on T cells are not suitable parameters for monitoring immunotherapy with an anti-idiotypic antibody because ACA125 provokes a rather weak (as shown by early activation events), but ongoing stimulus throughout the cycles of immunization.

In summary, this preliminary study could clearly prove a close relationship between anti-idiotypic vaccination and changes in intracellular IL-2, IL-4, and IFN-γ production, thus indicating the development of humoral and cellular immunity in immunized patients. The kinetics of Th1 and Th2 cytokine production under immunotherapy with ACA125 could be interpreted as an initial activation of the cellular branch of immunity followed by humoral immune responses. Whether the cellular or the humoral immune response is of predominant importance for antitumoral immunity remains unclear.

Our results demonstrate that measurement of Th1 and Th2 type cytokine production at the single T-cell level seems to be a rather simple and reliable parameter for monitoring immunotherapeutical effects.

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